




### AMENDMENT TO GLP TEST PROTOCOL

**Amendment No.:** 2  
**Effective Date:** November 8, 2016  
**Sponsor:** Reckitt Benckiser LLC  
Morris Corporate Center IV  
399 Interpace Parkway  
Parsippany, NJ 07054  
**Sponsor Representative:** Scientific & Regulatory Consultants, Inc.  
201 W. Van Buren Street  
Columbia City, IN 46725  
**Test Facility:** Accuratus Lab Services  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121  
**Protocol Title:** Disinfectant Efficacy Testing in the Presence of Organic Soil  
**Protocol Number:** SRC99072116.CUST.PROP  
**Project Number:** A21619


**Modifications to Protocol:**

Per Sponsor's request, the protocol is amended to change the exposure time from 5 minutes to 9.5 minutes for the repeat test of Formula 1178-172 Lots 2147-054 and 2147-086 using 100% plain cotton weave fabric carriers.

Changes to the protocol are acceptable as noted.

  
Study Director

11-9-16  
Date

  
Sponsor Representative

11-9-16  
Date

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INITIALS JLH DATE 12-13-16

**AMENDMENT TO GLP TEST PROTOCOL**

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**Amendment No.:** 1

**Effective Date:** September 30, 2016

**Sponsor:** Reckitt Benckiser LLC  
Morris Corporate Center IV  
399 Interpace Parkway  
Parsippany, NJ 07054

**Sponsor Representative:** Scientific & Regulatory Consultants, Inc.  
201 W. Van Buren Street  
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**Test Facility:** Accuratus Lab Services  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

**Protocol Title:** Disinfectant Efficacy Testing in the Presence of Organic Soil

**Protocol Number:** SRC99072116.CUST.PROP

**Project Number:** A21619

**Modifications to Protocol:**

Due to the many failed neutralization attempts with the test substances, this protocol is amended to allow for secondary neutralization as follows:

**Subculture and Neutralization of Treated Carriers**

1. Just prior to the contact time (e.g.  $\leq 10$  minutes) for the treated fabric test square, remove the lid or cap from the treatment vessel. At the contact time ( $\pm 5$  seconds), add 70 mL of neutralizer to the treatment vessel using a 100 mL sterile pipette. It is also acceptable to add a pre-measured 70 mL volume of neutralizer that was aliquoted prior to testing to the treatment vessel.
2. Replace the lid or cap of the treatment vessel.
3. Immediately vortex for 3-4 seconds each treatment vessel to ensure the treated test carrier is completely submerged in the neutralization media, and that the product overspray is thoroughly mixed with the neutralizer media.
4. Carriers will be transferred into individual secondary treatment vessels containing 70 mL of neutralizing subculture medium within approximately 25-60 minutes of the addition of the neutralizing subculture medium to the primary treatment vessels. Immediately vortex the treatment vessels for 3-4 seconds.
5. Place all of the treatment vessels in the incubator at the temperature appropriate for growth of the test organism. The growth temperature will be detailed in the protocol, raw data and final report.
6. Incubate for the time specified for the test organism.

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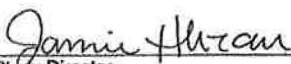
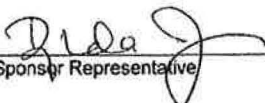
**AMENDMENT TO GLP TEST PROTOCOL**

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**Neutralization Confirmation Control**

1. The neutralization assay is performed using the same test substance parameters (dilution), test fabrics and treatment conditions (temperature, contact time).
2. For each organism dilution to be tested, treat two (2) sterile un-inoculated carriers with the test substance as described in the efficacy test. For example, 12 carriers would be treated when one test organism is assayed against, two fabric types, and three organism dilutions.
3. As in the efficacy assay, sterile fabric carriers are treated individually in treatment vessels.
4. At the contact time for each treated carrier, add 70 mL of the neutralizer media used in the efficacy test into the treatment vessel that contains the treated sterile carrier.
5. Each carrier will then be transferred from the primary treatment vessels into individual secondary treatment vessels containing 70 mL of neutralizer media beginning approximately 25-60 minutes following the addition of the neutralizing subculture medium to the primary treatment vessels.
6. Serially dilute the test organisms (no organic soil) out to the  $10^{-7}$  dilution or other appropriate dilution.
7. For each organism and organism dilution tested, inoculate four (4) treatment vessels (two primary and two secondary treatment vessels) with 0.1 mL of the organism dilution that will deliver between 10 and 100 organisms into the subculture tube.
8. Confirm the number of organisms initially delivered into the subculture tubes by plating 0.1 mL for each of the test culture dilutions in duplicate.
9. Incubate the neutralization assay test materials under the same conditions as previously described for the efficacy test. These conditions are specified in Table 1.
10. After the appropriate incubation duration, observe each treatment vessel for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media. Record the result for each treatment vessel as "+" for growth, or "0" for no growth.
11. Count and record the number of colonies on each agar plate.
12. Neutralization of the test substance by the neutralizer used in the efficacy study will be deemed acceptable if there is growth in both of the secondary treatment vessels containing the treated carriers, minimally, and the number of organisms introduced into each vessel is determined to be between 10-100 colonies as demonstrated on the corresponding agar plates. The neutralization assay can be repeated with the same batch of subculture neutralizing media used in the efficacy test if the number of organisms is determined to be out of this acceptable range (i.e. <10 or >100 CFU).

Changes to the protocol are acceptable as noted.

  
Study Director10-3-16  
Date  
Sponsor Representative10-4-16  
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(For Laboratory Use Only) <b>A21619</b> Accuratus Lab Services Project # <b>10P8-19-16</b>
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**ACCURATUS**  
LAB SERVICES

Test Substance Tracking #: **TS031416.SRC99**  
**KAM 7-21-16**

**PROTOCOL**

**Disinfectant Efficacy Testing in the Presence of Organic Soil**

**Test Organism(s):**

*Trichophyton mentagrophytes* (ATCC 9533)

**PROTOCOL NUMBER**

SRC99072116.CUST.PROP

**PREPARED FOR/SPONSOR**

Reckitt Benckiser LLC  
Morris Corporate Center IV  
399 Interpace Parkway  
Parsippany, NJ 07054

**SPONSOR REPRESENTATIVE**

Scientific & Regulatory Consultants, Inc.  
201 W. Van Buren Street  
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**PERFORMING LABORATORY/TESTING FACILITY**

Accuratus Lab Services  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

**DATE**

July 21, 2016

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INITIALS JLH DATE 12-13-16

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Protocol Number: SRC99072116.CUST.PROP

Reckitt Benckiser LLC  
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## Disinfectant Efficacy Testing in the Presence of Organic Soil

### PURPOSE

The purpose of this study is to demonstrate the disinfectant activity of the test substance against the specified test organisms as required for the desired claim on representative, soft, porous surfaces (i.e. cotton and polyester fabric).

### TEST SUBSTANCE CHARACTERIZATION

According to (40 CFR, Part 160, Subpart F [160.105]) test substance characterization as to identity, strength, purity, solubility and composition, as applicable, shall be documented before its use in this study. The stability of the test substance shall be determined prior to or concurrently with this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to Accuratus Lab Services. Accuratus Lab Services will append Sponsor-provided Certificates of Analysis (C of A) to this study report, if requested and supplied. Characterization and stability studies not performed following GLP regulations will be noted in the Good Laboratory Practice compliance statement.

### SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once Accuratus Lab Services receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is August 8, 2016. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of September 5, 2016. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at Accuratus Lab Services.

If a test must be repeated, or a portion of it, due to failure by Accuratus Lab Services to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test. Neither the name of Accuratus Lab Services nor any of its employees are to be used in advertising or other promotion without written consent from Accuratus Lab Services.

The Sponsor is responsible for any rejection of the final report by the regulatory agencies concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the Accuratus Lab Services final report and notify Accuratus Lab Services of any perceived deficiencies in these areas before submission of the report to the regulatory agency. Accuratus Lab Services will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

### JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The U.S. Environmental Protection Agency specifies that the AOAC Germicidal Spray Products or Use-Dilution Test are acceptable methods to determine efficacy for limited, general, hospital bacterial disinfection, pathogenic fungi or other microorganism claims for liquid/aerosol/trigger/pump spray product forms. This study will generate data to support a disinfectant efficacy claim against pathogenic fungi for soft surfaces based on the RB Confidential Protocol EPA File Symbol 777-PA-3, Disinfectant Efficacy Testing In the Presence of Organic Soil (May 19, 2015 / Version number 5) accepted by EPA on September 30, 2015.

#### **PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM**

Accuratus Lab Services maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including test organism strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subcultures, etc. during the course of the test. Test subcultures are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and/or macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

#### **ASSAY EVALUATION REQUIREMENTS:**

The success criteria for soft surfaces will be identical to the EPA requirements for hard surfaces in the AOAC Germicidal Spray Test (2013). The success criterion for fungicidal claims is:

Killing of the fungal spores on all of 10 carriers is required. The requirement for soft surfaces includes both natural and synthetic fabric types. Ten (10) carriers of each fabric type will be tested per test system per batch. Testing must be performed on each of 2 samples representing 2 different batches per fabric type per test strain.

#### **ASSAY ACCEPTANCE CRITERIA:**

The assay will be accepted for evaluating the test substance if the following criteria are satisfied:

1. The mean Log<sub>10</sub> density (LD) for fungal test systems must be at least 4.0 (corresponding to a geometric mean density of  $1.0 \times 10^4$  CFU/carrier) and not above 5.5 (corresponding to a geometric mean density of  $3.2 \times 10^5$  CFU/carrier).
2. The neutralizer must be shown to be effective, non-toxic, and support the growth of a low number of organisms (i.e. 10-100 CFU).
3. The identity of each test system must be verified on any day it is used for testing.
4. Growth must be present in the Test System Viability Controls.
5. The media, test surfaces and organic soil used in the study must be verified as sterile.

#### **Retesting Guidance**

1. For tests where the product passes and the mean Test LD value is above Log<sub>10</sub> 5.5 log/carrier, no retesting is necessary.
2. For tests where the product fails and the mean Test LD value is below Log<sub>10</sub> 4.0 log/carrier, no retesting is necessary.
3. For tests where the product fails and the mean Test LD value is above Log<sub>10</sub> 5.5 log/carrier, retesting may be conducted.

#### **AOAC Germicidal Spray Test Experimental Design/Operating Technique**

##### **Test System – Organisms**

The test organism(s) specified in Table 1 will be used in the assay. The test organism(s) was obtained from the American Type Culture Collection (ATCC), Manassas, VA

Table 1:

Organism	ATCC Number	Growth Media	Recovery Media	Incubation Parameters	Test Incubation Time
<i>Trichophyton mentagrophytes</i>	9533	Sabouraud Dextrose Agar	Glucose Agar	25-30°C	Broth 10-15 days Agar 66-76 hrs



#### Test Fabric Preparation

In this assay, 1 inch by 1 inch fabric squares will be used in place of the hard, non-porous surface (i.e. glass carriers) to serve as the test surfaces to demonstrate disinfection on a soft inanimate porous surface.

If claims are desired for cotton fabrics, then the fabric squares used for testing will be made of 100% cotton. If claims are desired for polyester fabrics, then the fabric squares used for testing will be made of 100% polyester. If general claims for fabric are desired, then both 100% cotton and 100% polyester fabric squares will be tested.

The test fabrics are described below:

1. Natural fabric -plain weave 100% cotton fabric. The fabric will be completely de-sized, bleached and without bluing or optical brighteners. The fabric is obtained from Test Fabrics Inc, West Pittston, Pennsylvania and is Style #400M.
2. Synthetic fabric – 100% polyester fabric. The fabric will be completely de-sized, bleached and without bluing or optical brighteners. The fabric is obtained from Test Fabrics Inc, West Pittston, Pennsylvania and is Style #777.

Prior to testing, the fabric will be processed in the following manner. If multiple fabric types are tested, each fabric type will be processed separately. The documentation of this processing will be recorded.

1. In a stainless steel pot, prepare a fabric scouring solution by adding 1.5 grams Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ ) and 1.5 grams Triton X-100 to 3 liters of deionized water. Equivalent dilutions may be made.
2. Add approximately 100 grams of test fabric per liter of scouring solution.
3. Allow the solution to reach a rolling boil. Boil for  $\geq 60$  minutes but  $\leq 70$  minutes.
4. Using tongs, remove the fabric from the scouring solution.
5. Rinse thoroughly with deionized water until all traces (foaming) of the wetting agents are visibly noted to be gone. This can be achieved by running the deionized water to re-fill the pot. To aid in removing the scouring solution, the fabric can be rung out occasionally with gloved hands.

**Note: Only gloved hands should be used when handling the fabric from this point forward. This will avoid adding unwanted body oils to the fabric.**

6. Allow the fabric to air dry completely by hanging or draping, the time taken for drying will be recorded.
7. Using scissors and a ruler, cut 1 inch x 1 inch test squares from the dried fabric.
8. Place each test square into separate glass petri dishes.
9. Steam sterilize.
10. Cool and store the fabric test squares at ambient temperature for  $\leq 30$  days.

#### Test System Preparation (Culture Transfer)

The maintenance and storage of stock cultures is described in detail in Accuratus Lab Services SOPs for culture maintenance (CGT-0020 and CGT-0070, current revisions). For testing of pathogenic fungi, a spore suspension will be utilized as the inoculum for testing in place of the consecutive broth to broth transfers described for bacteria in the EPA approved protocol.

#### *Trichophyton mentagrophytes*

A culture of *Trichophyton mentagrophytes* will be prepared by inoculating a sufficient number of Sabouraud Dextrose Agar plates using a stock culture and incubating at 25-30°C for 10-15 days. The mycelia will be removed from sufficient plates using a sterile device. The mycelia will be transferred to sterile glassware (e.g. an Erlenmeyer flask) containing glass beads and a ratio of 25 mL of sterile saline or saline/Triton Solution (0.85% Saline + 0.05 % Triton X-100) per 5 plates harvested. The culture will be agitated. Alternately, the mycelia may be added to a tissue grinder containing sterile saline or saline/Triton Solution and macerated. The culture will be filtered through sterile gauze to remove hyphal fragments. The conidial concentration will be estimated by counting in a hemacytometer and the culture may be adjusted as necessary. The inoculum must contain at least  $5 \times 10^6$  conidia/mL. The culture may be stored at 2-8°C for up to 4 weeks prior to use in testing.

**Final Culture Preparation:**

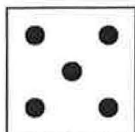
1. For the purpose of achieving the carrier count range, dilution of the final test culture may be performed as described above with sterile culture medium. Dilution of the final test culture should be made prior to the addition of the organic soil load to the inoculum.
2. Concentration of the final test culture may be necessary in the event the titer in the final test cultures is too low.
3. Concentration may be achieved using centrifugation, and re-suspending the pellet in the appropriate volume of the sterile culture medium to meet the carrier count range (at least  $5 \times 10^6$  conidia/mL).

**Preparation of Test Culture/Addition of Organic Load:**

1. Vortex the test culture for >3 seconds.
2. Determine the amount of culture that will be necessary for testing purposes.
3. Pipette an appropriate amount of culture into a sterile vessel and add a volume of blood serum sufficient to achieve a final serum concentration range of 5% (v/v). Any combination of culture/serum volumes which results in a final concentration of 5% serum is acceptable (i.e. 19.0 mL culture + 1.0 mL serum for 5%). This will be referred to as the TEST CULTURE.
4. Any unusual observations or problems with the test culture will be noted on the raw data sheet (e.g. clumping, debris, graininess), and reported to the Study Director immediately after it is observed or discovered.
5. Repeat for each of the test organisms.

**Fabric Test Surface/Carrier Inoculation**

1. Inoculate a total of 0.05 mL (50 µL) of the test culture onto each sterile carrier using a calibrated positive displacement micropipette.
2. Ten (10) microliters (µL) of the Test Culture will be inoculated to each of five areas of the fabric carrier (the total inoculum of the five areas being 0.05 mL) using the same pipet tip. Tips should be changed at least between every 5 carriers. Each corner and the direct center are the target areas. See diagram below:



3. For each fabric type, inoculate the appropriate number of test squares for each organism and batch to be tested.

For example:

- a. 10 fabric test squares per batch of the test substance for each fungal test system to be assayed (2 test substance batches) (1 test systems) = 20 fabric test squares per fabric type.
- b. Two (2) test squares for (2) test systems to serve as Test Viability Control = Four (4) fabric test squares per fabric type.
- c. Six (6) test squares for each of (2) test systems to serve as Dried Recovery Count Controls = Six (6) fabric test squares per fabric type per organism.
- d. Additional carriers (e.g. 3 per organism per fabric type) should be inoculated to serve as extras or reserves in the event that a carrier is accidentally dropped, or to assess if the inoculated carriers are sufficiently dry after the drying period. This is described in further detail in the following section.



#### **Drying the Inoculated Test Surface**

1. Place the petri dishes containing the inoculated carriers into a relative humidity (RH) incubator that maintains  $36 \pm 1^\circ\text{C}$  and dry for 30 to 40 minutes.
2. The carriers will be visually observed at the end of the drying period to determine if the test culture is dried. If additional drying time is required, this will be documented in the raw data. If a visual observation is not sufficient in determining whether a set of carriers is dry, an extra inoculated carrier can be used to blot the surface of a laboratory Kimwipe. A dried carrier will not transfer any culture to the Kimwipe (i.e. there will be no "wet spot" observed on the Kimwipe).
3. At least 6 extra carriers per fabric type per organism will be dried to perform the Dried Recovery Control. Three of the carriers will be selected randomly and assayed before the treatment portion of the assay is performed, and (3) carriers will be selected randomly assayed after the treatment portion of the assay is performed. The purpose of this control is to quantify the number of fungi surviving the drying process for each drying period.
4. Raw data entries at the time of testing will provide substantiation that the carriers were assayed before and after treatment as described above.
5. Use inoculated carriers within 2 hours of drying.

#### **Treatment of the Test System (Inoculated Carrier) with the Test Substance**

1. The ambient temperature of the room will be monitored and the maximum and minimum temperature recorded during the Treatment of the Test System and Subculture manipulations using an appropriate temperature monitoring device.
2. After drying, each inoculated and dried fabric swatch will be aseptically transferred from the glass petri dish to an individual sterile testing vessel. The inoculated side of the fabric swatch will be placed facing upward. The vessel will be re-capped until it is used in the efficacy test.
3. Prior to the treatment of an inoculated carrier, remove the lid from the treatment vessel.
4. Tilt the treatment vessel slightly (equal to or less than a  $45^\circ$  angle).
5. Treat the inoculated and dried fabric carrier with a batch of the test substance as per the sponsors use instructions (i.e. 2-3 second spray from a distance of 6-8 inches from the surface, 2-3 pumps from a distance of 6-8 inches from the surface).
6. Replace the treatment vessel lid to protect the test carrier from contamination.
7. At an appropriate time interval, treat the next carrier as described in the previous steps.
8. Continue with Steps 3 to 6 until all of the inoculated carriers for that treatment set have been treated.
9. Allow the product to remain in contact with each inoculated fabric test square for the specified contact time. The contact time starts at the time the product is first applied to the test square.

#### **Subculture and Neutralization of Treated Carriers**

1. Just prior to the contact time (e.g.  $\leq 10$  minutes) for the treated fabric test square, remove the lid or cap from the treatment vessel. At the contact time ( $\pm 5$  seconds), add 70 mL of neutralizer to the treatment vessel using a 100 mL sterile pipette. It is also acceptable to add a pre-measured 70 mL volume of neutralizer that was aliquoted prior to testing to the treatment vessel.
2. Replace the lid or cap of the treatment vessel.
3. Immediately vortex for 3-4 seconds each treatment vessel to ensure the treated test carrier is completely submerged in the neutralization media, and that the product overspray is thoroughly mixed with the neutralizer media.
4. Place all of the treatment vessels in the incubator at the temperature appropriate for growth of the test organism. The growth temperature will be detailed in the protocol, raw data and final report.
5. Incubate for the time specified for the test organism.

#### **Incubation and Observation of Subculture Tubes and Recording Results**

1. All subcultures are incubated as listed in Table 1.
2. Remove the test materials from the incubator after the appropriate incubation period.
3. Observe each treatment vessel containing a carrier for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media. Record the result for each treatment vessel as "+" for growth, or "0" for no growth.
4. Incubation at a temperature outside of the range listed in Table 1 will be considered a protocol deviation but will be deemed acceptable if the Dried Recovery control values are within the expected range ( $\geq 10^4$  organisms per carrier).

#### **Investigation of Presumptive Positives**

1. In the event the evaluation of any batch for any of the organisms tested demonstrates a presumptive positive (turbid, cloudy tube); an investigation of the results will be conducted on each tube to verify the presumptive positive results. The investigation will identify the growth through direct colony observation and Lactophenol Blue stain as compared to a control.
2. Results of the investigation will be reviewed by the Study Director and a conclusion will be drawn as to the identity of the presumptive positive, the acceptance or rejection of the results obtained, and any necessary next steps (e.g. re-test due to the confirmed presence of a contaminant).
3. Growth that is confirmed to be the test organism is not allowable in any of the 10 carriers treated (i.e. a result of 0/10 is a passing result). Confirmed growth in 1 or more treatment vessel that is determined to be a contaminant (i.e. growth that was investigated, and concluded not to be the test system), will invalidate the assay.

#### **STUDY CONTROLS**

##### **Verification of the Identity of the Test Cultures**

The identity of each test organism will be verified in parallel with the test per the following instructions.

1. On the day of testing, the stock culture of each test organism will be streaked for isolation on an appropriate growth agar and incubated along with the test and other controls per Table 1.
2. Following incubation, the identity of the organism will be verified by observing the colony morphology and performing a Lactophenol Cotton Blue Stain.
3. Observations from these procedures will be compared to the known characteristics of each test organism as listed in Accuratus Lab Services SOP CGT-0067, current revision.
4. Results of this verification will be reviewed by the Study Director. The identity of the test system will be accepted or rejected based upon the criteria provided in the SOP. This conclusion will be recorded in the raw data. If the test system verification is rejected, the assay will be considered invalid and the test will be terminated. All test materials will be discarded.

##### **Survivor Counts/Dried Recovery Control Carriers**

The purpose of this control is to determine the number of viable organisms that remain on the inoculated test carriers after the drying period. Six (6) carriers, per test organism, per fabric type, are inoculated and dried as described previously in the test procedure. Dried recovery carriers are inoculated and dried along with efficacy carriers, and are selected randomly. Recovery counts are performed on each dried recovery control carrier as described below.

1. As stated previously, 3 of the inoculated dried recovery control carriers will be dried and assayed before the treatment or efficacy portion of the assay is conducted, and 3 will be assayed after the treatment or efficacy portion of the assay is completed.
2. After drying, and at the appropriate subculture time, using sterile forceps, subculture each inoculated and dried recovery control carrier into a treatment vessel that contains 70 mL of neutralizing media and 10 grams glass beads. Vortex each treatment vessel containing a dried recovery control carrier for 120±5 seconds.
3. Prepare tenfold or hundredfold dilutions from the treatment vessel containing each carrier in subculture media or an appropriate diluent. The treatment vessel containing the carrier is considered the  $10^0$  dilution.
4. Plate in duplicate 1.0 mL or 0.1 mL aliquots of the  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilution for each recovery control carrier with the appropriate media for the test organism.
5. Incubate the dried recovery control plates under the same conditions as previously described in the efficacy test. These conditions are specified in Table 1.
6. After the appropriate incubation duration, count the colonies on each plate and determine the number of organisms surviving on the carrier as per the instructions provided in the calculations section of this protocol.



#### Test System Viability Control Carriers

The purpose of this control is to confirm viable organisms that remain on the inoculated test carriers after the drying period. Two (2) carriers per test organism, per fabric type, are inoculated and dried as described previously in the test procedure.

1. After drying, individually subculture the two (2) inoculated test system viability control carriers into separate treatment vessels that contain 70 mL of the subculture media that was used in the efficacy assay.
2. Incubate the treatment vessels under the same conditions as previously described in the efficacy test. These conditions are specified in Table 1.
3. After the appropriate incubation duration, observe each treatment vessel for the presence of growth. Growth or turbidity in each of the two (2) treatment vessels per test organism is considered an acceptable result for this control assay

#### Neutralization Confirmation Control

1. The neutralization assay is performed using the same test substance parameters (dilution), test fabrics and treatment conditions (temperature, contact time).
2. For each organism dilution to be tested, treat two (2) sterile un-inoculated carriers with the test substance as described in the efficacy test. For example, 12 carriers would be treated when one test organism is assayed against, two fabric types, and three organism dilutions.
3. As in the efficacy assay, sterile fabric carriers are treated individually in treatment vessels.
4. At the contact time for each treated carrier, add 70 mL of the neutralizer media used in the efficacy test into the treatment vessel that contains the treated sterile carrier.
5. Serially dilute the test organisms (no organic soil) out to the  $10^{-7}$  dilution or other appropriate dilution.
6. For each organism and organism dilution tested, inoculate two (2) treatment vessels (containing the treated carrier and overspray) with 0.1 mL of the organism dilution that will deliver between 10 and 100 organisms into the subculture tube.
7. Confirm the number of organisms initially delivered into the subculture tubes by plating 0.1 mL for each of the test culture dilutions in duplicate.
8. Incubate the neutralization assay test materials under the same conditions as previously described for the efficacy test. These conditions are specified in Table 1.
9. After the appropriate incubation duration, observe each treatment vessel containing a carrier for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media. Record the result for each treatment vessel as "+" for growth, or "0" for no growth.
10. Count and record the number of colonies on each agar plate.
11. Neutralization of the test substance by the neutralizer used in the efficacy study will be deemed acceptable if there is growth in both of the treatment vessels containing the treated carriers, and the number of organisms introduced into each vessel is determined to be between 10-100 colonies as demonstrated on the corresponding agar plates. The neutralization assay can be repeated with the same batch of subculture neutralizing media used in the efficacy test if the number of organisms is determined to be out of this acceptable range (i.e. <10 or >100 CFU).

#### Media Sterility Control

1. For liquid media (i.e. broth) at least one un-inoculated tube of each batch of liquid media used in the assay is incubated under the same conditions as stated in the efficacy assay.
2. For agars, at least one additional plate of each batch of agar media is left un-inoculated, and allowed to incubate under the same conditions as stated in Table 1.
3. Record any growth on a particular batch of media as "NS" for non-sterile, and no growth as an "S" for sterile.
4. If a particular batch of media demonstrates growth, and the test substance demonstrates efficacy, the identity of the test organism in each of the tubes and on the plates in the Neutralizer Efficacy assay must be verified.
5. If it is determined that the growth in the Neutralization assay is the test organism, the assay will be accepted.
6. A second sterility test on another tube or agar plate can also be performed. In event that similar growth is observed, in the second sterility test, the Study Director will be notified and a scientifically valid rationale will be developed for accepting/rejecting the results of the test using this media.



#### Test Surface (Carrier) Sterility Control

1. One un-inoculated test surface (carrier) will be added to a subculture tube containing the neutralizing media used in the efficacy study.
2. The subculture tube will be incubated under the same conditions as listed in Table 1.
3. Record growth in the subculture tube as "NS" for non-sterile, and no growth as an "S" for sterile.
4. If the test surface fails (i.e. growth in subculture tube) the sterility test, and the test substance passes efficacy testing, the identity of the test organism in each of the tubes and on the plates in the Neutralizer Efficacy assay must be verified. If it is determined that the growth in the Neutralization assay is the test organism, the assay will be accepted.

#### Organic Soil Sterility Control

1. A 0.1 mL aliquot of the organic soil used in the study will be inoculated into a subculture tube containing 10 mL of neutralizing or growth media.
2. The subculture tube will be incubated under the same conditions as listed in Table 1.
3. Record growth in the subculture tube as a "NS" for non-sterile" and no growth as an "S" for sterile.
4. The Study Director will be informed of any non-sterile results and a scientifically valid rationale will be developed for accepting/rejecting the results of the test using this organic soil.

#### Test System Inoculum Count

1. Dilute each test system (without organic soil) to the  $10^{-7}$  dilution or other appropriate dilution.
2. Plate 1.0 mL aliquots of the  $10^{-8}$  and  $10^{-7}$  dilutions (or other applicable dilutions) in duplicate with the appropriate growth agar.
3. Incubate the agar plates under the same conditions as previously described for the efficacy test. These conditions are specified in Table 1.
4. After the appropriate incubation duration, count and record the number of colonies of each agar plate.

#### DATA ANALYSIS

##### Calculations

1. Colony Forming Units (CFU) per mL (or organisms per mL)

CFU/mL = A x B/C where

A= Dilution Factor. The dilution factor is the inverse of the serial dilution used.

B= Number of colonies per plate

C= Volume plated

2. Mean Log Density (M) of Dried Recovery Counts

- a. Count and record the number of colonies appearing on each plate. Record counts >300 as TNTC.
- b. For each replicate (duplicate plating), using values of 0 to 300, determine the 'Average Plate Count per Dilution' for each dilution plated. Round to the nearest whole number. If duplicate platings of a dilution yield results of 1 and 0, the average of these values (0.5) will be rounded to the nearest whole number (1).
- c. For each replicate, determine the 'Average CFU / mL' as per the following equation:

$$\text{Average CFU/mL} = \frac{(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})}{(10^{-x}) + (10^{-y}) + (10^{-z})}$$

where  $10^{-x}$ ,  $10^{-y}$ , and  $10^{-z}$  are example dilutions that may be used

- d. For each replicate, determine the 'Average CFU / carrier' by multiplying the 'Average CFU / mL' by the volume of subculture medium in the test tubes containing the Dried Recovery Count carriers
- e. For each replicate, determine the Log Density by calculating the  $\text{Log}_{10}$  value of the 'Average CFU / carrier'.
- f. Determine the Mean  $\text{Log}_{10}$  Density (M) by averaging the individual Log Densities.
- g. Round results to provide 3 significant figures (e.g.  $3.29 \times 10^4$  or  $4.52 \text{ Log}_{10}$ ).

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**Statistical Analysis**  
None Used

**METHOD FOR CONTROL OF BIAS:** NA

**RECORD RETENTION**

**Study Specific Documents**

All of the original raw data developed exclusively for this study shall be archived at Accuratus Lab Services for a minimum of five years for GLP studies or a minimum of six months for all other studies following the study completion date. After this time, the Sponsor (or the Sponsor Representative, if applicable) will be contacted to determine the final disposition. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited, to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study specific SOP deviations made during the study.

**Facility Specific Documents**

The following records shall also be archived at Accuratus Lab Services. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

**REPORT**

The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the organism strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

**PROTOCOL CHANGES**

If it becomes necessary to make changes in the approved protocol, the revision and reasons for change will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the current effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.



#### **TEST SUBSTANCE RETENTION**

**It is the responsibility of the Sponsor to retain samples of the test substance. All unused test substance will be discarded following study completion unless otherwise requested.**

#### **REFERENCES**

1. Official Methods of Analysis of the AOAC International, Official Method 961.02, Germicidal Spray Products as Disinfectants, Chapter 6, Revised First Action, 2012.
2. Official Methods of Analysis of the AOAC International, Official Method 955.14, 955.15 and 964.02, Use-Dilution Method, Revised 2013, Chapter 6.
3. Official Methods of Analysis of the AOAC International, Official Method 955.17, Fungicidal Activity of Disinfectants, Chapter 6.
4. Disinfectant Technical Science Section (DIS/TSS) Efficacy Documents, Disinfectants for use on hard surfaces, DIS/TSS – 1, January 22, 1982
5. Disinfectant Technical Science Section (DIS/TSS) Efficacy Documents, Supplemental Recommendations, DIS/TSS – 2, January 25, 1979
6. Disinfectant Technical Science Section (DIS/TSS) Efficacy Documents, Confirmatory Efficacy Data Requirements, DIS/TSS – 5, September 22, 1982
7. Disinfectant Technical Science Section (DIS/TSS) Efficacy Documents, Supplemental Efficacy, DIS/TSS – 6.
8. US EPA OCSPP 810.2200: Disinfectants for Use on Hard Surfaces – Efficacy Data Recommendations. September 4, 2012.
9. US EPA OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents. September 4, 2012.
10. US EPA OCSPP 810.2400: Disinfectants and Sanitizers for Use on Fabrics and Textiles – Efficacy Data Recommendations. December 21, 2012.
11. Petrocci, A.N. and Clarke, P. Proposed Test Methods for Antimicrobial laundry Additives. Journal of the AOAC Vol.52. No.4, pp. 836-842, 1969.
12. ASTM Standards Vol. 11.05, E 2274-09 Standard Test Method for Evaluation of Laundry Sanitizers and Disinfectants.
13. ASTM Standards Vol. 11.05, E 1153-14 Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces.
14. Reckitt Benckiser Confidential Protocol EPA File Symbol 777-PA-3, Disinfectant Efficacy Testing In the Presence of Organic Soil (May 19, 2015 / Version number 5). Accepted September 30, 2015.
15. Official Methods of Analysis of the AOAC International, Official Method 972.04 Bacteriostatic Activity of Laundry Additive Disinfectants, First Action 1972, Chapter 6.



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#### STUDY INFORMATION

(All blank sections are completed by the Sponsor or Sponsor Representative as linked to their signature, unless otherwise noted.)

**Test Substance (Name & Batch Numbers) exactly as it should appear on final report:**

Formula 1178-172, lot 2147-054 and Lot 2147-086

#### Product Description:

- |  |  |
|--|--|
| <input checked="" type="checkbox"/> Quaternary ammonia | <input type="checkbox"/> Peracetic acid                  |
| <input type="checkbox"/> Iodophor                      | <input type="checkbox"/> Peroxide                        |
| <input type="checkbox"/> Sodium hypochlorite           | <input checked="" type="checkbox"/> Other <u>Ethanol</u> |

**Approximate Test Substance Active Concentration (upon submission to Accuratus Lab Services):**

<0.09% quat, <53% Ethanol

(This value is used for neutralization planning only. This value is not intended to represent characterization values.)

**Neutralization/Subculture Broth:** ☐

(NOTE: All broth must also serve as an appropriate growth medium for the test organism)

- ☒ Accuratus Lab Services' Discretion. By checking, the Sponsor authorizes Accuratus Lab Services, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

#### Storage Conditions:

- ☒ Room Temperature  
☐ 2-8°C  
☐ Other: \_\_\_\_\_

#### Hazards:

- ☐ None known: Use Standard Precautions  
☒ Material Safety Data Sheet, Attached for each product  
☐ As Follows: \_\_\_\_\_

#### Product Preparation

- ☒ No dilution required, Use as received (RTU)  
☐ \*Dilution(s) to be tested: \_\_\_\_\_ defined as \_\_\_\_\_ + \_\_\_\_\_  
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)  
☐ Deionized Water (Filter or Autoclave Sterilized)  
☐ Soft Tap Water (Filter or Autoclave Sterilized) - All tap water is softened; the water hardness for the batch of tap water used will be determined and reported.  
☐ AOAC Synthetic Hard Water: \_\_\_\_\_ PPM  
☐ Other: \_\_\_\_\_

**\*Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.**

**Test Organism(s):** ☒ *Trichophyton mentagrophytes* (ATCC 9533)

**Fabric Carrier Type:** ☒ 100% Plain Cotton Weave ☐ Other: \_\_\_\_\_  
☒ 100% Polyester

**Carrier Number:** 10 per batch

**Spraying Time or # of Sprays:** 3 seconds or until thoroughly wet

**Approximate Spraying Distance:** 6-8 inches

**Exposure Time:** 5 Minutes **Exposure Temperature:** Room temperature

#### Organic Soil Load:

- ☒ Minimum 5% Organic Soil Load (Fetal Bovine Serum)  
☐ No Organic Soil Load Required  
☐ Other: \_\_\_\_\_

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#### **SPRAY BOTTLES USED IN TESTING**

*To ensure expected levels of product are delivered, it is recommended that the Sponsor provide the spray bottles used in testing. Please indicate the desired source of the sprayer bottles used in testing:*

- ☒ Sprayer(s) and bottle(s) are provided by the Sponsor
- ☐ General purpose spray bottle(s) are to be provided by Accuratus Lab Services
- ☐ The spray nozzle(s) are provided by the Sponsor and general purpose bottle(s) will be provided by Accuratus Lab Services

#### **TEST SUBSTANCE SHIPMENT STATUS**

*(This section is for informational purposes only.)*

- ☒ Test Substance is already present at Accuratus Lab Services.
- ☐ Test Substance has been or will be shipped to Accuratus Lab Services.

Date of expected receipt at Accuratus Lab Services: \_\_\_\_\_

- ☐ Test Substance to be hand-delivered (must arrive by noon at least one day prior to testing or other arrangements made with the Study director)

#### **COMPLIANCE**

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.

- ☒ Yes
- ☐ No (Non-GLP or Development Study)

#### **REGULATORY AGENCY(S) THAT MAY REVIEW DATA**

- ☒ U.S. EPA
- ☐ Health Canada
- ☐ Therapeutic Goods Administration (Australian TGA)

#### **PROTOCOL MODIFICATIONS**

- ☐ Approved without modification
- ☒ Approved with modification

The subcultures will not be refrigerated after incubation. A draft report will be provided for review prior to finalization. Insert "EPA Reg. No. 777-99" in report header. Test Fabric Preparation: If after drying, the fabric is wrinkled, the fabric may be ironed flat so that flat swatches may be prepared. Steam will not be used. Calculations section 2.d. the average CFU/carrier will be calculated by also dividing by the volume plated."

#### **PROTOCOL ATTACHMENTS**

Supplemental Information Form Attached - ☐ Yes ☒ No

**TEST SUBSTANCE CHARACTERIZATION & STABILITY TESTING**

[Verification required per 40 CFR Part 160 Subpart B (160.31(d))].

☐ Characterization/Stability testing is not required (For Non-GLP or Development testing only)

**OR**

Physical and Chemical Characterization (Identity, purity, strength, solubility, as applicable) of the test lots

☒ **Physical & Chemical Characterization has been or will be completed prior to efficacy testing.**

GLP compliance status of physical & chemical characterization testing:

☒ Testing was or will be performed following 40 CFR Part 160 GLP regulations

☐ Characterization has not been or will not be performed following GLP regulations

Check and complete the following that apply:

☒ A Certificate of Analysis (C of A) may be provided for each lot of test substance. If provided, the C of A will be appended to the report.

☐ Testing has been or will be conducted at Accuratus Lab Services under protocol or study #:

☐ Test has been or will be conducted by another facility under protocol or study #:

☐ **Physical & Chemical Characterization was not or will not be performed prior to efficacy testing.**

Stability Testing of the formulation

☒ **Stability testing has been or will be completed prior to or concurrent with efficacy testing.**

GLP compliance status of stability testing:

*(GLP compliance is required by 40 CFR Part 160)*

☒ Testing was or will be performed following 40 CFR Part 160 GLP regulations

☐ Stability testing has not been or will not be performed following GLP regulations

Check and complete the following that apply:

☐ Testing has been or will be conducted at Accuratus Lab Services under protocol or study #:

☐ Test has been or will be conducted by another facility under protocol or study #:

☐ **Stability testing was not or will not be performed prior to or concurrent with efficacy testing.**

*If test substance characterization or stability testing information is not provided or is not performed following GLP regulations, this will be indicated in the GLP compliance statement of the final report.*





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**ACCURATUS**  
LAB SERVICES

**APPROVAL SIGNATURES**

**SPONSOR:**

NAME: Ms. Rhonda Jones TITLE: Agent  
SIGNATURE: [Signature] DATE: 7-21-16  
PHONE: (260) 344 - 6270 FAX: (260) 244 - 6273 EMAIL: rjones@srcconsultants.com

*For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.*

**Other individuals authorized to receive information regarding this study:**

☐ See Attached

Diane Boesenberg, Lisa Dreilinger, SRC staff, Shahper Rahman

**Accuratus Lab Services:**

NAME: Jamie Herzan  
Study Director  
SIGNATURE: [Signature] DATE: 8-18-16  
Study Director

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